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Protein tyrosine phosphorylation in signalling pathways leading to the activation of gelatinase A: activation of gelatinase A by treatment with the protein tyrosine phosphatase inhibitor sodium orthovanadate

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Abstract

Fibroblasts in monolayer culture secrete gelatinase A (MMP2; 72 kDa type IV collagenase) only in its proenzyme form. Unlike other secreted matrix metalloproteinases, progelatinase A is refractory to activation by serine proteinases. Disparate agents, including monensin, cytochalasin D, and concanavalin A, have been found to mediate the activation of gelatinase A zymogen secreted by fibroblast monolayers. Our finding that monensin-mediated activation can be reversed by the protein tyrosine kinase inhibitor genistein (Li et al., Experimental Cell Research 232 (1997) 332) prompted us to investigate the effect of the specific inhibitor of protein tyrosine phosphatases, sodium orthovanadate, on progelatinase A activation. Treatment of fibroblast monolayers with orthovanadate also results in the secretion of activated gelatinase A. This activation is dose- and time-dependent, requires protein synthesis, and is associated with cell membranes. Vanadate-mediated activation does not occur in the presence of herbimycin A, a protein tyrosine kinase inhibitor. As with progelatinase activation mediated by monensin, concanavalin A, and cytochalasin D, orthovanadate treatment results in increased synthesis of the membrane proteinase MT1-MMP, that can catalyze the activation of progelatinase A. Protein tyrosine kinase inhibitors are able to prevent the increase of MT1-MMP mRNA, as shown by Northern blot and RT-PCR. In addition, orthovanadate potentiates the effects of monensin and concanavalin A. While treatment with monensin or concanavalin A result only in an increase of the putative activator MT1-MMP, orthovanadate also reduces the production of the specific inhibitor TIMP-2. These experiments implicate protein tyrosine phosphorylation in the signal transduction pathways which lead to the activation of progelatinase A.

Keywords: Tyrosine phosphorylation; Gelatinase A activation

1. Introduction

The matrix metalloproteinase gelatinase A (MMP2; 72 kDa type IV collagenase) is thought to play a major role in extracellular matrix degradation during both normal and pathological processes, especially in tumor invasion and metastasis [1–3]. Like all matrix metalloproteinases (MMPs), gelatinase A is synthesized as an inactive zymogen and converts to its active form by removal of the amino-terminal pro-peptide. Unlike all other secreted MMPs, gelatinase A is refractory to activation by limited proteolysis catalyzed by serine proteinases and other MMPs

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[4,5]. Although gelatinase A is found in a wide variety of human cell types and is implicated in a number of disease processes, the physiological mechanisms leading to its activation remain unclear.

Activation of gelatinase A at the cell plasma membrane has been suggested by several studies [3,6]. This hypothesis is strengthened by the discovery of a membrane-type matrix metalloproteinase (MT1-MMP) that is able to activate gelatinase A [6]. Agents with different physical and chemical characteristics, such as the lectin concanavalin A [7], the sodium ionophore monensin [8], and the cytoskeletal-disrupting agent cytochalasin D [9,10] have been found to stimulate activation of progelatinase A in monolayer cultures of normal fibroblasts. In addition, treatment of fibrosarcoma cells and endothelial cells with the tumor promoter phorbol myristic acetate [11,12] results in progelatinase A activation. In all these cases, MT1-MMP is upregulated [8,10,12–14]. The exception is activation of gelatinase A by fibroblasts cultured in a type I collagen lattice. In this case, activation occurs intracellularly and is associated with Golgi-enriched intracellular membranes [15,16]. MT1-MMP is not increased in collagen lattice culture, but may well be the activating agent in the Golgi, where MT1-MMP itself appears to be converted from its zymogen form [15,16]. Production of gelatinase A protein is increased in this system, as it is with concanavalin A treatment of fibroblast monolayers [7].

We have previously shown [8] that monensin induces a membrane associated activation of gelatinase A in normal human skin fibroblasts grown in monolayer culture. The monensin effect on gelatinase A activation is accompanied by an increase in MT1-MMP production and is inhibited by the protein kinase inhibitor genistein [8]. These results suggest a role for protein tyrosine phosphorylation (PTP) in the signal transduction pathways leading to activation of gelatinase A. Because tumor severity and metastatic potential have been shown in many cases to be correlated with the extent of gelatinase A activation [17,18], it is of great interest to further investigate the role of PTP in this process.

In this paper, we report that sodium orthovanadate (vanadate), an inhibitor of tyrosine phosphatases [19], can itself induce a membrane-associated activation of gelatinase A in monolayer cultures of skin fibroblasts. Vanadate also potentiates the effects of other activating agents, such as monensin or concanavalin A. Gelatinase A activation by vanadate, as well as monensin and concanavalin A, can be reversed by certain protein tyrosine kinase inhibitors, such as herbimycin A. These results support the hypothesis that protein phosphorylation is involved in the pathway leading to the activation of progelatinase A by MT1-MMP.

2. Materials and methods

2.1. Materials

Sodium orthovanadate (vanadate), cycloheximide (CHX), genistein, gelatin, bovine albumin and concanavalin A were purchased from Sigma (St. Louis, MO). Lavendustin A was purchased from LC Laboratories (Woburn, MA). Herbimycin A and monensin were obtained from Calbiochem (La Jolla, CA). Alkaline phosphatase-conjugated anti-chicken, anti-mouse and anti-rabbit antibodies were purchased from Sigma. TIMP-2 antibody was purchased from Triple Point Biologics (Forest Grove, OR). Monoclonal antibody to MT1-MMP was a kind gift from Dr. H. Seiki (Kanazawa University, Japan). TIMP-2 was purified from culture medium produced by a recombinant cell line (generous gift from Dr. Gregory Goldberg, Washington University School of Medicine) by established protocols [11].

2.2. Cell culture

Human fibroblast cell strains were established from normal adult skin by outgrowth from explants as previously described [20]. Cultures were maintained with DME/F12 medium containing a mixture of 5% newborn calf serum (Biowhittaker) and 3% bovine embryonic fluid (Sigma). All cultures of normal fibroblast cells used in this study were between passages 3 and 11. In order to exclude interference resulting from latent and active gelatinase A existing in serum and embryonic fluid, experiments were performed in serum free DME/F12 medium containing insulin-transferrin-selenium (ITS) culture supplement (Collaborative Biomedical Products). ITS medium has been previously shown to be an adequate

serum substitute for several days of serum-free culture [21].

Cell viability was measured with the colorimetric cytotoxicity method of Alley et al. [22].

2.3. Fibroblast populated collagen lattices

Collagen lattices were prepared using sterile type I collagen from rat tail tendons as previously described [15]. Concentrations of collagen in the lattice varied from 0.5 to 1 mg/ml. Lattice mixtures were made in pH-adjusted DME/F12 so that the culture conditions were identical to the control monolayer plates. Collagen-containing mixtures were kept at 4°C until the addition of cells. After mixing, aliquots of 1–2 ml were pipetted into 35 mm bacteriological dishes that had been precoated with bovine serum albumin to prevent fibroblast adhesion. Gelation began immediately and was completed in less than 2 min at 37°C, trapping the cells within the lattice. Cultures were incubated at 37°C in an atmosphere of 95% air, 5% CO_2. After 1 h, lattices were detached from the sides of the dish by gentle tapping.

2.4. Zymogram analysis of proenzyme activation

Latent and active gelatinases were detected by zymogram analysis using SDS-polyacrylamide gels copolymerized with 2.5% gelatin as previously described [23]. These enzymes become dissociated from TIMP's by the presence of SDS during electrophoresis. Removal of SDS following electrophoresis allows the proenzymes to renature in an active or partially active conformation. This permits their detection and the detection of lower molecular weight activated forms [24,25]. In brief, conditioned medium or whole cell lysate was mixed with sample buffer and electrophoresed directly without boiling or reduction. Following electrophoresis, SDS was extracted from the polyacrylamide gel with Triton X-100, and the gel was incubated in 0.05 M Tris, pH 7.5, containing 5 mM $CaCl_2$, and 5 μM $ZnCl_2$ at 37°C overnight. Gels were stained with Coomassie blue and destained. Both proenzyme and active gelatinase were detected as clear bands against the blue background of the stained gelatin.

2.5. Preparation of whole cell lysate and subcellular fractionation for cellular activation of biosynthetically labeled gelatinase A

Normal fibroblasts were grown to confluence and treated with 100 μM vanadate in the presence or absence of 10 μg/ml cycloheximide (CHX) under serum-free conditions for 48 h. Following treatment, conditioned medium was removed and retained, and the cells were fractionated into a crude cell membrane fraction and a cytosolic fraction as described by Brown et al. [26]. The cells were incubated with lysis buffer containing 50 mM Tris-HCl pH 7.5, 0.2 M NaCl, 0.2% (v/v) Brij-35 and protease inhibitors including 1 μg/ml leupeptin (Sigma, L-2884), 10 μg/ml pepstatin A (Sigma, P-4265), 10 μg/ml aprotinin (Sigma, A-1153) and 1 mM pefabloc (Boehringer Mannheim) for 30 min at 4°C. After vigorous vortexing, this whole cell lysate was used for zymogram analysis.

For preparation of crude cell membranes and cytosolic fractions, the initial cell lysate was centrifuged at $2000 \times g$ for 30 min at 4°C. The supernatant was collected as the cytosolic fraction, and the cell residue resuspended in lysis buffer as a crude membrane fraction. Protein content was determined by Bradford's method [27] with bovine serum albumin as standard.

In order to determine which fraction is involved in the activation of progelatinase A, 20 μg of cytosol or crude membrane protein, and 20 μl of concentrated conditioned medium (15-fold concentration) were incubated with 5 or 10 μl of ^{35}S-labeled gelatinase A at 37°C for 48 h. The samples were then separated on 12% SDS-polyacrylamide gels, and the gels dried and processed for autoradiography. ^{35}S-Labeled gelatinase A used in this study was prepared and purified as previously described [23].

2.6. Western immunoblotting analysis

Conditioned medium and cell extracts were electrophoresed on 10 or 12% SDS-polyacrylamide gels, and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore) following the product directions. After blockage of non-specific

binding sites with non-fat milk, blots were incubated 1 h at room temperature with primary antibody. After extensive washing, blots were incubated with alkaline phosphatase-conjugated second antibodies, and developed with BCIP-NBT (Kirkegaard-Perry). The primary antibody for immunostaining of gelatinase A was raised in chickens and affinity purified using a column of gelatinase A coupled to Affi-gel 10. A polyclonal rabbit antibody to a unique peptide was used for immunodetection of TIMP-2, and a monoclonal anti-peptide antibody to MT1-MMP was used in Western blotting analysis of that enzyme.

2.7. Detection of tyrosine-phosphorylated proteins by Western immunoblotting

Normal fibroblasts were treated with vanadate in serum-free DME/F12 medium. At indicated time points, the medium was discarded and the cell layer rapidly rinsed with HBSS. The cell layer was then lysed with 0.5 ml lysis buffer containing 1% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 mM vanadate, 100 mM NaF, 1 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 1 mM pefabloc. The lysate was briefly sonicated, centrifuged at $14\,000\times g$ for 25 min to remove insoluble material, and assayed for protein concentration using the BCA protein reagent (Pierce). 20–30 μg of sample protein was mixed with sample buffer containing 10 mM DTT, boiled 5 min, and subjected to SDS-PAGE and electrophoretic transfer to a polyvinylidene difluoride membrane (PVDF membrane). After nonspecific binding sites were saturated with blocking buffer (1% bovine albumin in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween), the membranes were incubated 1 h at room temperature with horseradish peroxidase conjugated anti-phosphotyrosine (RC20, Transduction Laboratories, Lexington, KY). After extensive washing, tyrosine-phosphorylated proteins were detected with ECL reagents (Amersham).

2.8. Quantitation of MT1-MMP mRNA

2.8.1. Total cellular RNA preparation and Northern blot analysis

Cells were cultured in the presence of vanadate (100 μM) for 24 h. Total cellular RNA was extracted with the Trizol Kit (Gibco-BRL). 20 μg of total RNA was denatured at 95°C for 3 min and separated on formaldehyde–1% agarose gels, transferred to Nylon membranes by standard capillary blotting, and fixed at 80°C. Northern blot analysis was performed with cDNA probes labeled with [α-^{32}P]dCTP (Amersham, 3000 Ci/mmol) using Klenow enzyme. The MT1-MMP cDNA probe was a gift from Dr. Gregory Goldberg (Washington University). Quantitation was performed by direct densitometric measurement of the bands seen on X-ray films using the NIH Image (version 1.61).

2.8.2. RT-PCR analysis

RT-PCR was performed according to the protocol in the Perkin-Elmer GenAmp RNA PCR kit with the following modifications: the first strand cDNA was synthesized following kit instructions, but using 2 or 3 μg of total RNA and 200 units of M-MLV (Gibco) in a total volume of 20 μl. Reverse transcription proceeded for 1 h at 42°C, followed by a 5 min incubation at 99°C to inactivate M-MLV. After cooling to 4°C for 5 min the samples were kept on ice for PCR.

The sense and anti-sense primers used for MT1-MMP were, respectively, 5′-CTCTCTTCTGGATGCCCAAT-3′ and 5′-CCTCAATGATGATCACCTCC-3′, giving a PCR product of 350 bp [28]. GAPDH was used as an internal standard [29,30], with the sense primer 5′-ACTCTGGTAAAGTGGATATTG-3′ and the antisense primer 5′-TCCCGTTCAGCTCAGGGATGA-3′, giving a PCR product of 567 base pairs. Both MT1-MMP and GAPDH genes were amplified in one reaction tube. Amplification was carried out in a 20 μl volume using the Perkin–Elmer GeneAmp PCR system 2400 with a program consisting of 95°C for 2 min to denature followed by 45 cycles of 95°C for 15 s and 60°C for 30 s (anneal-elongation), and a final 10 min extension at 72°C. Following amplification, samples were electrophoresed on 1% agarose gels containing ethidium bromide in 1×TAE buffer. Gels were photographed under UV light using the Kodak Digital Science 1D Image Analysis system and bands quantitated by direct densitometric measurement using NIH Image (version 1.61).

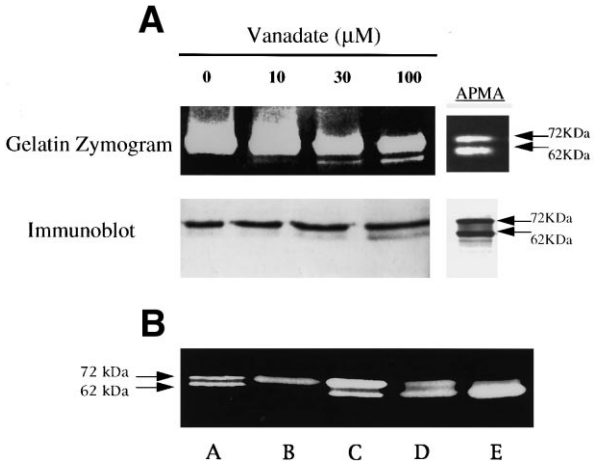

Fig. 1. Orthovanadate induces activation of progelatinase A in monolayer cultures of normal human dermal fibroblasts. (A) Dose response showing activation of progelatinase A with increasing concentrations of orthovanadate. Confluent fibroblasts were incubated with increasing doses of vanadate in ITS-serum free medium for 48 h. Medium was collected and directly subjected to zymography as described in Section 2. For immunoblot analysis, aliquots containing equal amounts of protein from concentrated medium were electrophoresed and transferred. Development of the immunoblot was performed as described in Section 2. Medium from a gelatinase A overexpression clone P211A [55] was incubated with 1 mM APMA at room temperature for 2 h and used as a positive control of active gelatinase A. (B) Comparison of gelatinase A activation in normal human fibroblasts mediated by different agents. Equal amounts of cells were incubated with the agents indicated for 48 h. Medium was directly subjected to zymography. (A) Medium from ITS-serum-free (ITS-SF) control cultures incubated with 1 mM APMA for 2 h at room temperature. (B) Medium from ITS-serum-free monolayer culture. (C) Medium from fibroblast monolayer culture in ITS-SF medium containing 100 μM vanadate. (D) Medium from type I collagen lattice culture. (E) Medium from fibroblast monolayer cultured in ITS-SF medium containing 20 μg/ml concanavalin A.

3. Results

3.1. Orthovanadate (vanadate) induces 72 kDa gelatinase A activation in human dermal fibroblasts

Normal human dermal fibroblasts in monolayer culture secrete only the proenzyme of gelatinase A. However, zymograms of conditioned medium from vanadate-treated monolayer cultures showed an induction of active gelatinase A in a dose-dependent manner (Fig. 1A). This induction was confirmed by immunoblotting using polyclonal anti-gelatinase A antibody (Fig. 1A). The extent of gelatinase A activation induced by vanadate is comparable to that produced by such agents as concanavalin A or culturing fibroblasts in a type I collagen lattice (Fig. 1B).

3.2. Vanadate-induced gelatinase A activation is associated with cell membranes and requires protein synthesis

As shown in Fig. 2, incubation of exogenous ^{35}S-labeled progelatinase A with a total cell membrane fraction (including ER, Golgi and plasma membranes) prepared from vanadate-treated fibroblasts showed that only membranes prepared from vanadate-treated cultures (lane C, Fig. 2) result in a marked activation of exogenous proenzyme. Membranes prepared from control monolayer cultures (lane B, Fig. 2) did not show activation. Neither the cytosolic fraction nor concentrated conditioned medium was able to activate labeled gelatinase A (data not shown). Membranes isolated from cultures incubated with 100 μM vanadate and 10 μg/ml cycloheximide, a protein translation inhibitor (lane D, Fig. 2), did not activate exogenous proenzyme, suggesting that protein synthesis is involved in vanadate-mediated activation of progelatinase A.

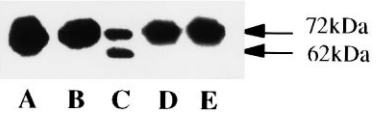

Fig. 2. Cell membrane associated activation of gelatinase A by vanadate and the effect of cycloheximide on activation. Fibroblasts were cultured for 48 h in ITS-SF medium ±100 μM vanadate and/or 10 μg/ml cycloheximide. Cells were harvested, separated into crude cytoplasmic and cell membrane fractions as described in Section 2. Twenty μg of membrane protein was then incubated with ^{35}S metabolically labeled pure gelatinase A proenzyme for 48 h at 37°C before electrophoresis and autoradiography. (A) ^{35}S-Progelatinase A alone. (B) ^{35}S-Progelatinase A+membranes from monolayer control fibroblasts. (C) ^{35}S-Progelatinase A+membranes from monolayer fibroblasts incubated with 100 μM vanadate. (D) ^{35}S-Progelatinase A+membranes from monolayer fibroblasts incubated with both 100 μM vanadate and 10 μg/ml cycloheximide. (E) ^{35}S-Progelatinase A+membranes from monolayer fibroblasts incubated with 10 μg/ml cycloheximide.

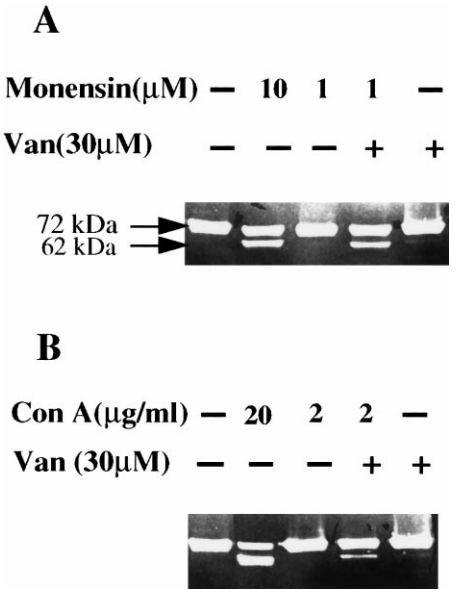

Fig. 3. Zymograms showing that vanadate acts synergistically with other gelatinase A activating agents. (A) Monensin was added to monolayer fibroblast cultures for 48 h at an activating concentration and at a concentration which did not give detectable activation by zymography. When vanadate at a suboptimal dose was included with the low dosage of monensin, activation of gelatinase A was detected. (B) An identical experiment to (A), but using low concentrations of concanavalin A.

3.3. *Vanadate acts synergistically with other agents that activate gelatinase A*

We have previously shown that the protein tyrosine kinase inhibitor genistein prevents monensin-induced activation of gelatinase A in fibroblast monolayers [8]. Since vanadate inhibits protein tyrosine phosphatases, and thus potentiates the action of tyrosine kinases, it was of interest to see if it would potentiate the action of agents mediating the activation of gelatinase A. For this purpose, very low doses (1/10 optimal) of monensin or concanavalin A were added to fibroblast cultures together with a low concentration of vanadate. After a 48 h incubation, medium was collected and analyzed by gelatin zymography. As shown in Fig. 3A, neither 30 μM vanadate nor 1 μM monensin alone is sufficient to mediate activation of gelatinase A. However, when monolayer cultures were incubated with both 30 μM vanadate and 1 μM monensin, considerable activation was observed. Similar results were obtained with 30 μM vanadate, and a concentration of concanavalin A which is 1/10 that needed for optimal activation (2 μg/ml) (Fig. 3B). Quantitation of both pro- and active bands using NIH Image 1.61 software clearly exhibit a marked increase of active gelatinase A in cells treated with both agents (not shown). These results show that vanadate, an inhibitor of protein tyrosine phosphatase, not only induces gelatinase A activation itself, but also potentiates the effect of other agents, again indicating the involvement of protein tyrosine phosphorylation in the pathways leading to activation of gelatinase A.

3.4. *The protein tyrosine kinase inhibitor herbimycin A decreases activation of gelatinase A*

To further address involvement of protein tyrosine phosphorylation in the activation of gelatinase A by fibroblasts in monolayer culture, the effects of protein tyrosine kinase inhibitors were investigated. Of the inhibitors tested (genistein, herbimycin A, and lavendustin A), only herbimycin A was able to inhibit activation in both systems (Fig. 4). Genistein was

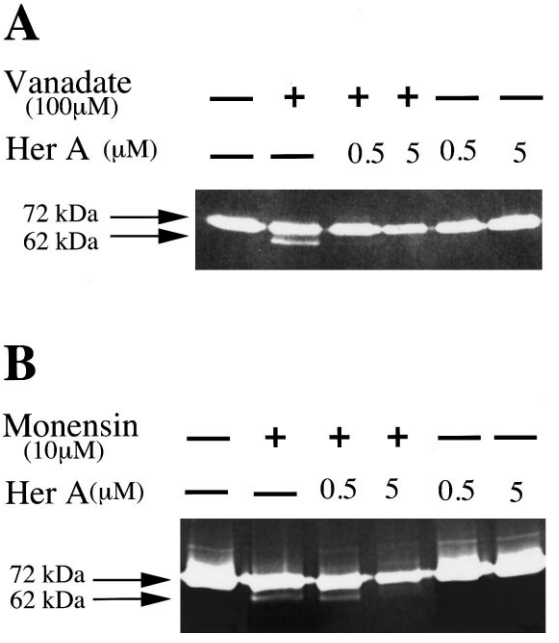

Fig. 4. Inhibition of protein tyrosine phosphorylation by herbimycin A inhibits progelatinase A activation by vanadate and monensin. Dermal fibroblasts were cultured in ITS-serum free medium containing either 100 μM orthovanadate (A) or 10 μM monensin (B). The indicated concentrations of herbimycin A were added at the beginning of the incubation. Medium was collected after 30 h and subjected to gelatin zymography.

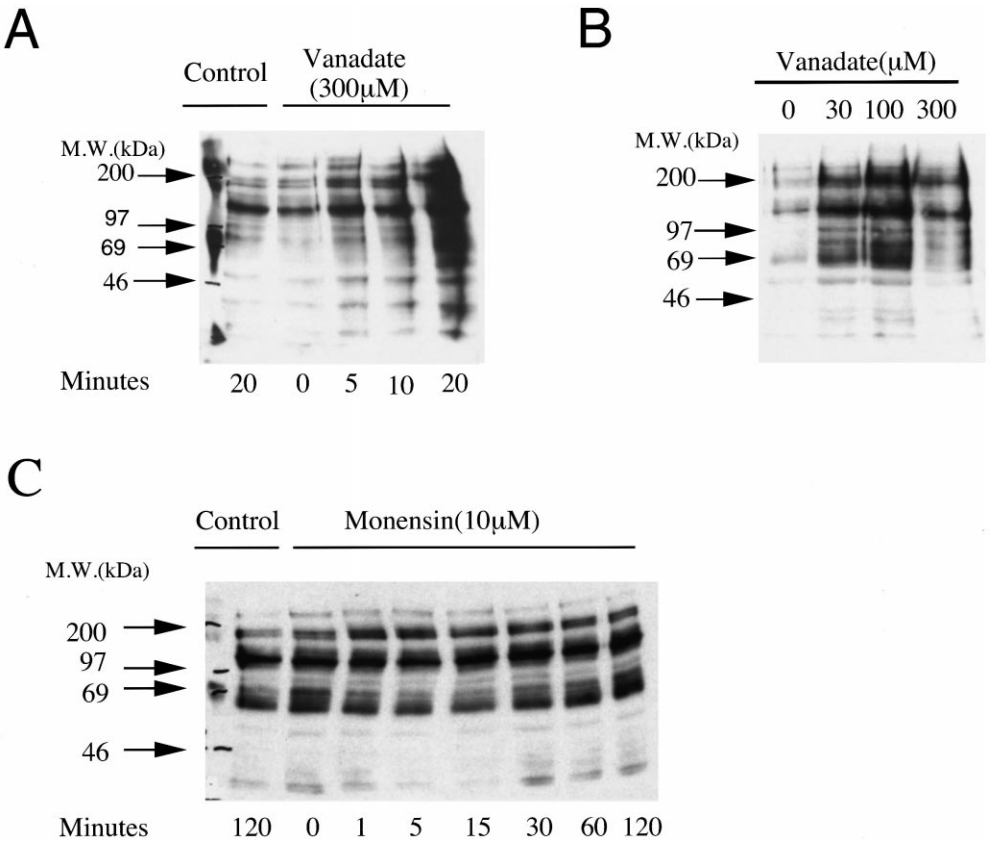

Fig. 5. Tyrosine phosphorylation of cell proteins from vanadate- and monensin-treated fibroblasts. Fibroblast cultures were exposed to monensin and vanadate as indicated. Tyrosine-phosphorylated proteins were detected by horseradish peroxidase conjugated anti-phosphotyrosine antibody (RC20, Transduction Laboratories) as described in Section 2. (A) Time course of tyrosine phosphorylation in cells exposed to 300 μM vanadate (V). The control was incubated in ITS-serum free medium for 20 min. (B) Dose response showing phosphorylation in fibroblasts exposed to ITS-serum-free medium containing increasing concentration of vanadate for 15 min. (C) Time course of tyrosine phosphorylation in cells exposed to 10 μM monensin. The control was incubated in ITS-serum free medium for 120 min.

able to reverse monensin-mediated activation of gelatinase A in monolayer culture, and lavendustin A reversed the activation mediated by vanadate (not shown). These results indicate that inhibition of protein tyrosine kinases can result in inhibition of gelatinase A activation.

3.5. *Patterns of tyrosine phosphorylation in cellular proteins were observed in fibroblasts in response to monensin and vanadate treatment*

Western immunoblots showed an increase in tyrosine phosphorylation of specific proteins in response to both vanadate and monensin treatment (Fig. 5). As expected, vanadate treatment resulted in accumulation of phosphorylated proteins in a dose-dependent and time-dependent fashion (Fig. 5A,B). Several of these bands, at approximately 190 and 130 kDa, appeared to be the same as bands phosphorylated in response to monensin treatment (Fig. 5C).

3.6. *Effect of orthovanadate on MT1-MMP expression*

Northern blots of total RNA from normal fibroblasts treated with 100 μM orthovanadate for 24 h showed a nearly two-fold increase in mRNA for MT1-MMP, a membrane-bound matrix metalloproteinase that can activate progelatinase A (Fig. 6A).

RT-PCR was used to compare the production of MT1-MMP mRNA by fibroblasts in response to orthovanadate, monensin, and concanavalin A with or

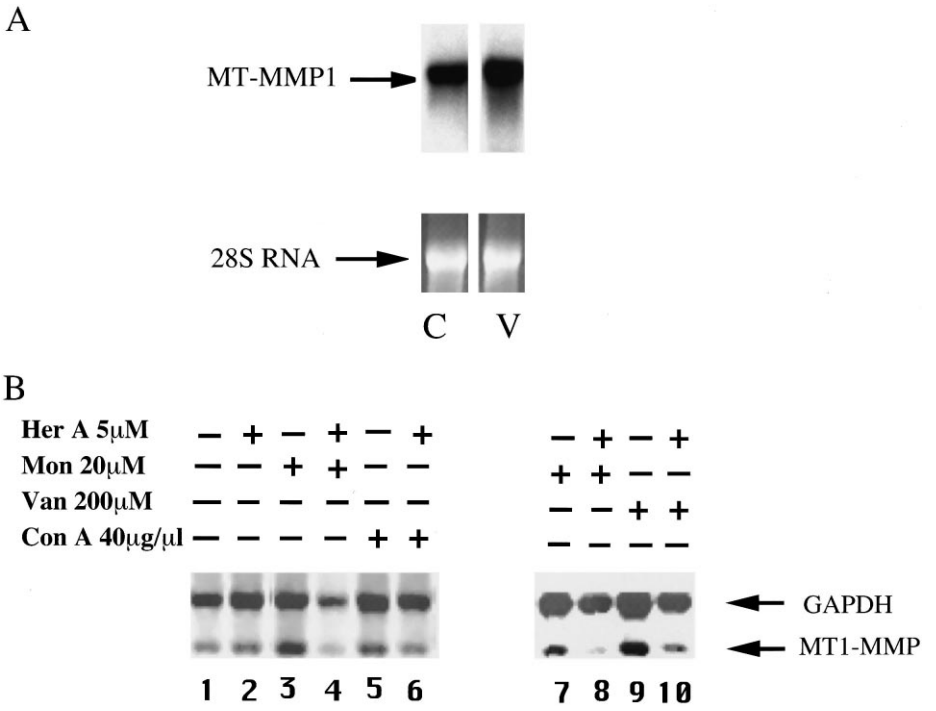

Fig. 6. Increase in MT1-MMP mediated by orthovanadate, and reversal of MT1-MMP induction by inhibitors of tyrosine phosphorylation. (A) Northern blot analysis of total RNA from fibroblast monolayer cultures. C, control; V, 100 μM vanadate (B) Confluent monolayer fibroblasts were incubated with combinations of herbimycin A (Her A), monensin (Mon), orthovanadate (Van) and concanavalin A (Con A) for 24 h in ITS serum free medium. Total RNA was then isolated and subjected to RT-PCR as described in Section 2. GAPDH, which was amplified with MT1-MMP in the same tube, was used as an internal standard.

without herbimycin A. As shown in Fig. 6B, treatment of monolayer cultures with vanadate (lane 9), monensin (lanes 3 and 7), and concanavalin A (lane 5) resulted in increased production of MT1-MMP mRNA. In all cases, when 5 μM herbimycin A was added to the incubations with the above agents (lanes 4, 6, 8 and 10), MT1-MMP mRNA remained near baseline levels, indicating that inhibition of protein tyrosine phosphorylation inhibits the upregulation of this enzyme by the agents investigated. Fig. 6B shows the results from two different experiments. The decrease in monensin-mediated MT1-MMP mRNA shown in lanes 7 and 8 confirms the results shown in lanes 3 and 4.

3.7. Coordinated increase in MT1-MMP and decrease in TIMP-2 is found in response to orthovanadate treatment of fibroblast monolayers

Immunoblot analysis of cell lysates showed that upregulation of the message for MT1-MMP resulted in increased synthesis of the protein. Quantitation of the Western immunoblot using NIH Image 1.61 showed a five-fold increase in both pro- and active MT1-MMP protein in monolayer cultures treated with vanadate (Fig. 7A), further implicating specific protein synthesis in the response to the phosphatase inhibitor. This increase in the putative gelatinase-activating enzyme is accompanied by a concurrent decrease in tissue inhibitor of metalloproteinase-2 (TIMP-2) in culture medium, as shown in Fig. 7B. A decrease in TIMP-2, which is often secreted in a complex with progelatinase A could indicate an increased amount of uncomplexed, and theoretically readily activatable proenzyme to serve as a substrate for MT1-MMP.

4. Discussion

Since inhibitors of matrix metalloproteinases have been shown to have anti-invasive and anti-metastatic effects [31,32], and since overexpression of TIMPs, the protein inhibitors of MMPs, also have anti-

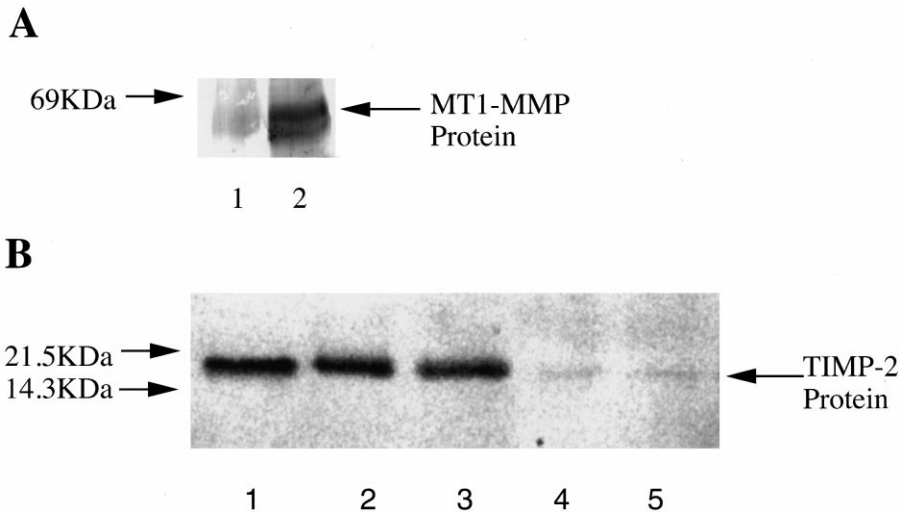

Fig. 7. Western blots display a coordinated increase of MT1-MMP and decrease of TIMP-2 in response to vanadate treatment. Confluent monolayers of fibroblasts were incubated with 100 μM vanadate in ITS-serum free medium for 48 h. Medium was collected and the cell layer was lysed for immunoblotting of MT1-MMP using a monoclonal antibody. Equal amounts of cell protein were electrophoresed prior to electrotransfer. Equal amounts of medium protein were electrophoresed and immunoblotted for TIMP-2 using a peptide-antibody. (A) Immunoblot for MT1-MMP: Lane 1, Control cell lysate; lane 2, cell lysate from 100 μM vanadate cultures. The two bands correspond to pro- and active enzyme. (B) Immunoblots for TIMP-2: Lane 1, purified TIMP-2; lanes 2 and 3, 25 μg medium protein from control cultures; lanes 4 and 5, 25 μg medium protein from cultures incubated with 100 μM vanadate.

metastatic effects [33,34], it can be concluded that active gelatinase A is the important form of the enzyme in disease processes. In many cases, direct correlations have been made between tumor aggression and extent of gelatinase A activation [17,18,35–37]. Elucidation of mechanisms leading to the production of active gelatinase A is thus of major importance.

Previously, we have shown that gelatinase A activation mediated by monensin can be prevented by incubation with the protein tyrosine kinase inhibitor genistein [8]. Here we show that orthovanadate, which inhibits dephosphorylation catalyzed by protein tyrosine phosphatases, can induce a dose-dependent, cell membrane-associated activation of gelatinase A. Furthermore, vanadate is able to potentiate the effects of other agents that mediate activation of gelatinase A and apparently act via pathways involving protein tyrosine phosphorylation. Inhibitors of protein tyrosine kinases prevent vanadate-mediated and monensin-mediated activation of progelatinase A. Indeed, Western blotting for protein tyrosine phosphate indicates that several of the proteins phosphorylated in response to monensin also correspond to bands showing increased phosphorylation when normal fibroblasts are treated with orthovanadate. The identity of these bands is under investigation.

Incubation of fibroblast monolayers with monensin, vanadate, or concanavalin A results in an increase of MT1-MMP, a membrane-bound metalloproteinase that can activate progelatinase A. The results shown in this study indicate that protein tyrosine kinase signal transduction cascades are involved in the up-regulation of this protein. Further support comes from the PCR experiments in which MT1-MMP mRNA levels were maintained at baseline when 5 μM herbimycin was included in the presence of an activating concentration of monensin, vanadate, or concanavalin A. However, despite the similarities between monensin- and vanadate-mediated activation of progelatinase A, the signalling pathways involved must differ, since only vanadate treatment results in decreased production of the specific protein inhibitor TIMP-2. This dual target may explain the synergistic effect of vanadate and other activating agents. The potentiation of monensin and concanavalin A activation of gelatinase A by vanadate might be due to the availability for activation of a greater percentage of total proenzyme not complexed to TIMP-2. Earlier work showed that the TIMP-2-progelatinase A complex is more difficult

to activate than uncomplexed proenzyme [18,38]. Subsequent experiments showed that TIMP-2 can actually potentiate progelatinase A activation when present in a very precise ratio to MT1-MMP, but that a very slight increase in the TIMP-2:MT1-MMP ratio will inhibit activation [11,39]. The experiments described in this paper do not permit the quantitation that would be required to accurately interpret the role of reduced TIMP-2 production in this system.

It should be pointed out that in the case of integrin-mediated progelatinase A activation in the type I collagen lattice, we have previously shown that MT1-MMP and TIMP-2 levels are unchanged, while production of gelatinase A itself is up-regulated [15,16]. Clearly, similar end results can be achieved by altering the relationships between the components of the system; i.e. the proenzyme, the activator, and the inhibitor.

The agents that mediate activation of progelatinase A are seemingly quite disparate in their properties. For example, monensin is a sodium ionophore, while concanavalin A is a lectin. However, all of the agents investigated to date, including the mechanically relaxed collagen lattice [40–42], produce major changes in the cytoskeletal organization. We have found striking, but quite different morphologic changes when normal fibroblasts were exposed to monensin, vanadate, and concanavalin A in monolayer cultures (data not shown). These changes are correlated with marked changes in the actin cytoskeleton, as shown by immunofluorescent staining with phalloidin. It is well documented that tyrosine phosphorylations play a crucial role in the regulation of cytoskeletal functions [43–46]. Components of the cytoskeleton play a role in passing signals from the extracellular environment to the nucleus to regulate gene expression [47–49]. For instance, binding of extracellular matrix molecules to their integrin receptors elicits tyrosine phosphorylation of the cytoskeletal molecules paxillin and tensin [50,51], initiating a cascade of signals that alter expression of many genes. Furthermore, Tomasek et al. [40] have shown that the organization of the polymerized actin cytoskeleton is an important factor in the regulation of gelatinase A activation.

Matrix metalloproteinase inhibitors have been shown to be effective anti-tumor and anti-metastatic agents in cell culture and animal models [31,33,34,37,52]. Clinical trials in humans are in the early stages, and have been somewhat promising [31,53,54]. Because of the strong correlations often observed between tumor aggressiveness and extent of gelatinase activation, it is essential to dissect the pathways leading to such activation. The experiments described in this paper indicate that protein tyrosine phosphorylations are involved in at least some of these pathways.

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